

Exonuclease Domain of the Lassa Virus Nucleoprotein Is Critical To Avoid RIG-I Signaling and To Inhibit the Innate Immune Response

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Lassa virus (LASV), which causes a viral hemorrhagic fever, inhibits the innate immune response. The exonuclease (ExoN) domain of its nucleoprotein (NP) is implicated in the suppression of retinoic acid-inducible gene I (RIG-I) signaling. We show here that a LASV in which ExoN function has been abolished strongly activates innate immunity and that this effect is dependent on RIG-I signaling. These results highlight the key role of NP ExoN function in the immune evasion that occurs during LASV infection.

Lassa virus (LASV) is an Old World arenavirus responsible for a hemorrhagic fever, which affects 300,000 to 500,000 people annually and causes about 6,000 deaths (1). This virus is a major public health concern in West Africa. Lassa fever varies from asymptomatic infections to fatal hemorrhagic fever (2). Dendritic cells (DC) and macrophages (MΦ) are the principal targets of infection (3, 4). DC infection leads to a massive release of LASV, without the induction of cell activation and cell maturation or the production of cytokines. Similarly, MΦ are productively infected but display no sign of activation other than modest type I interferon (IFN) production (4, 5). In contrast, Mopeia virus (MOPV), a nonpathogenic arenavirus closely related to LASV (6, 7), rapidly activates MΦ but induces only a slight activation of DC, which produce modest amounts of type I IFN (8). The ways in which LASV and, to a lesser extent, MOPV inhibit the activation of DC and MΦ are unknown. Several residues of the arenavirus nucleoprotein (NP), including D389 and G392, have been implicated in the inhibition of type I IFN production (9–11). This inhibition is due to the 3′–5′ exonuclease (ExoN) function of the NP, which digests free double-stranded RNA. This prevents RNA recognition by retinoic acid-inducible gene I (RIG-I), a pattern recognition receptor (PRR) that recognizes double-stranded RNA (12–15). We used our reverse-genetics system for LASV to generate a recombinant wild-type LASV (LASV-WT) and another LASV, LASV-ExoN, with two mutations (D389A and G392A) affecting the NP (16). As previously described by several groups, each of these mutations is sufficient to completely inhibit exonuclease activity (12, 13, 15). The infection of DC and MΦ with LASV-ExoN resulted in the production of much larger amounts of type I IFN, CC chemokines, and CXC chemokines than infection with LASV-WT (16, 17). In this study, we characterized the responses of antigen-presenting cells (APC) to infection with this virus and assessed the role of RIG-I in these responses. All experiments with viruses were performed in biosafety level 4 (BSL4) facilities (Jean Mérieux, INSERM BSL4, Lyons, France).

We first analyzed the activation profiles of DC and MΦ derived from human monocytes, during infections with LASV-ExoN, LASV-WT, and MOPV. DC were not activated in response to infection with LASV and MOPV but were strongly activated in response to infection with LASV-ExoN. They expressed large amounts of the costimulatory molecules implicated in T-cell acti-

vation, such as CD86, CD80, and CD40, but also of CD83, an indicator of DC maturation (Fig. 1A). MΦ infection with MOPV and, to a lesser extent, LASV-WT resulted in activation. However, LASV-ExoN activated MΦ more strongly, resulting in the expression of large amounts of costimulatory molecules. Surprisingly, CD83 was also expressed by MΦ, probably due to the very high level of activation, as already reported in other studies (18). We previously described replication of LASV-WT and LASV-ExoN (16). LASV-ExoN infection is productive in VeroE6 cells, and large amounts of virus are released in supernatants. In contrast, replication of LASV-ExoN in APC is attenuated, suggesting that DC and MΦ activation is correlated with the control of viral replication. Previous results showed that LASV-ExoN-infected APC secrete large amounts of type I IFN (16). The role of the type I IFN response in the control of replication has to be elucidated. However, we were not able to block type I IFN response using different neutralizing antibodies, as the level of IFN was too high and the remaining molecules still induced cell activation and induction of interferon-stimulated gene synthesis (data not shown). The expression of some molecules involved in the induction of the adaptive immune response was also studied. Among them, DC-LAMP and HLAabc were overexpressed on LASV-ExoN-infected DC (Fig. 1B) and may favor an efficient adaptive immune response. Interestingly, the active form of caspase 3 was present in large amounts in LASV-ExoN-infected DC and MΦ (Fig. 1C), suggesting that the infection by LASV-ExoN leads to the apoptosis of APC. Indeed, the active form of caspase 3, obtained after the cleavage of procaspase 3, is specific to apoptosis. The abolition of NP ExoN function may thus result in a change in the fate of LASV-infected APC. It remains unclear whether this phenomenon occurs downstream

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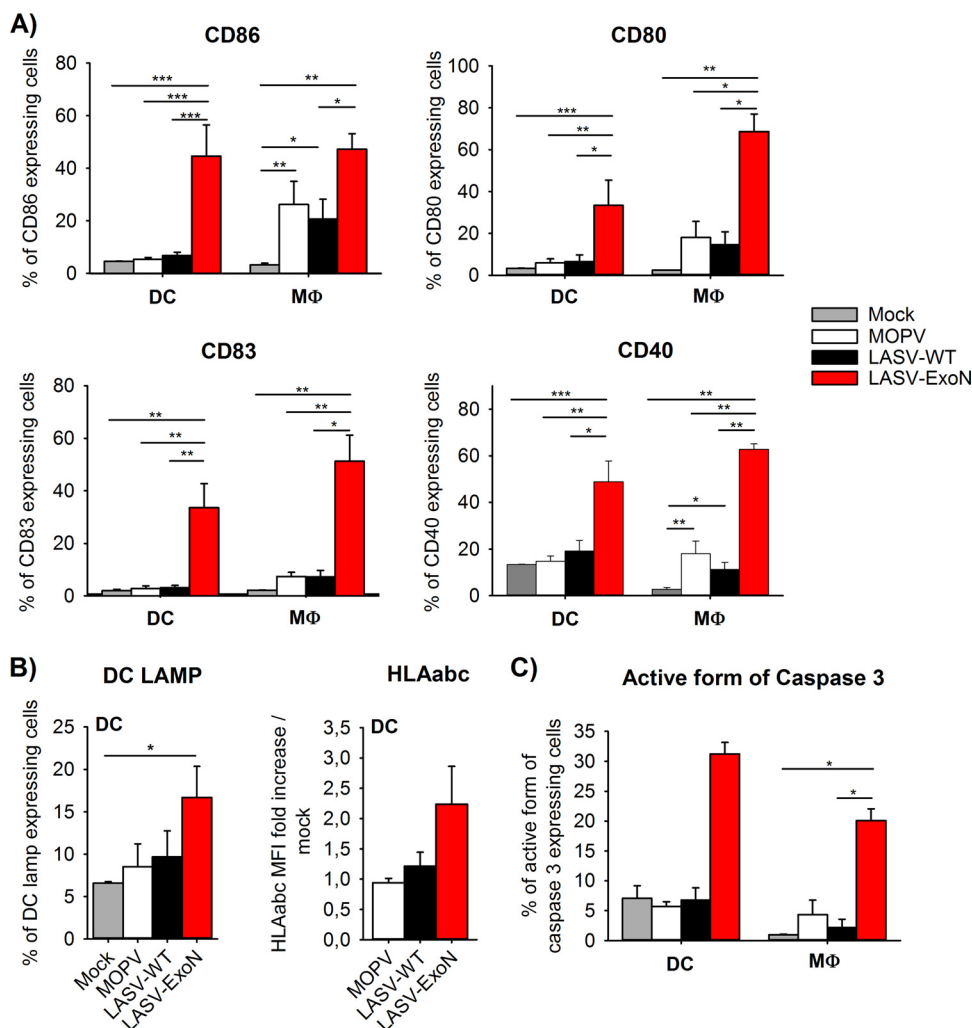


FIG 1 Phenotype of DC and MΦ after infection with MOPV, LASV-WT, or LASV-ExoN. DC and MΦ were derived from monocytes isolated from the peripheral blood of healthy donors, as previously described (4). Cells were infected at a multiplicity of infection (MOI) of 2 and analyzed 24 h after infection. They were stained with antibodies (a list is available upon request) as previously described (24) and then analyzed with a Gallios cytometer and Kaluza software (Beckman Coulter). (A) Expression of activation markers on DC and MΦ. Results are expressed as the means \pm standard errors (SE) of results from 6 or 7 experiments with cells from different donors. (B) Expression in DC and MΦ of markers involved in the adaptive immune response in DC (DC LAMP and HLAabc). Experiments were carried out on cells from 3 to 5 different donors, and the results are expressed as means \pm SE. (C) Expression of the active form of caspase 3 in DC and MΦ. Experiments were carried out on cells from 3 or 4 different donors, and the results are expressed as means \pm SE. Mann-Whitney tests were carried out for statistical analysis. Significant differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

of cell activation and IFN response or is due to another mechanism triggered by the virus itself. It has recently been described that NP of Junin virus, a new world arenavirus, is remarkably involved in the inhibition of apoptosis during infection by being cleaved as a bait instead of caspases (19). Other data suggest that arenaviruses may inhibit apoptosis via the binding of Z protein to promyelocytic leukemia (PML) protein (20).

The robust activation of DC and MΦ observed during LASV-ExoN infection led us to analyze the mRNA profile of the cells. We previously reported that the infection of DC and MΦ with LASV-ExoN resulted in very high levels of mRNA synthesis for type I IFN and chemokines (16, 17). We show here that infection with LASV-ExoN leads to the synthesis of mRNA for tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and IL12p35 (Fig. 2). In contrast, LASV-WT and MOPV did not

promote the synthesis of these transcripts in DC and induced only low levels of proinflammatory cytokine mRNA synthesis in MΦ, essentially for MOPV.

Unlike LASV-WT, LASV-ExoN, which has lost 3'-5' exonuclease activity (12, 13, 15), strongly activated DC and MΦ, suggesting an important role for ExoN in immunosuppression. The role of the RIG-I pathway in this inhibition remains unclear and was then investigated.

It has been shown that transfected LASV RNA activates the beta IFN (IFN- β) promoter via RIG-I but not MDA-5 (14). However, we investigated the role of RIG-I and MDA-5 in LASV infection. We determined the levels of mRNA in DC and MΦ infected with MOPV, LASV-WT, or LASV-ExoN. Surprisingly, similar levels of mRNA synthesis were observed in MΦ for all the viruses tested (Fig. 3). In contrast, in DC, MDA-5 mRNA synthesis seemed to be induced in response to each virus, but it was signif-

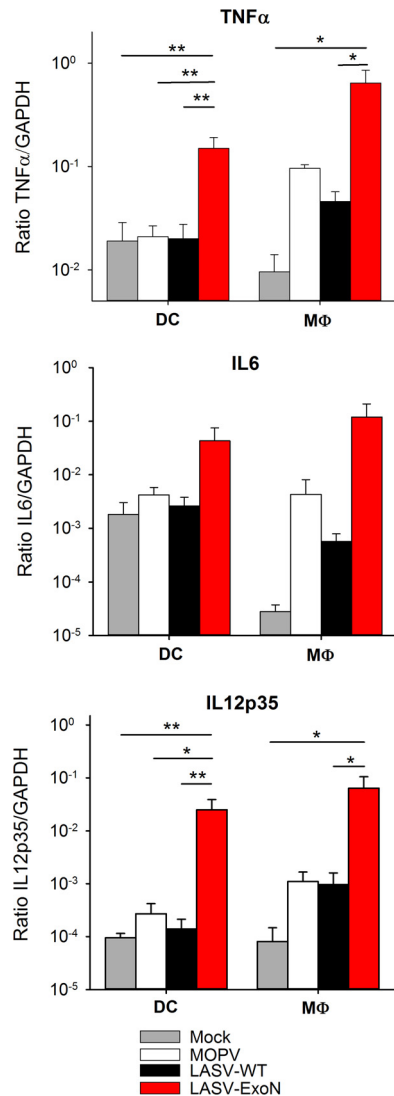


FIG 2 Cytokine production by infected DC and MΦ. Total RNA was extracted from cells 24 h after infection (MOI of 2). cDNA was synthesized with oligo(dT) primers and amplified by quantitative PCR, as previously described. Results are expressed as gene/GAPDH (glyceraldehyde-3-phosphate dehydrogenase) ratios and as the means \pm SE of results from 4 to 6 experiments performed with cells from different donors. Mann-Whitney tests were carried out for statistical analysis. Significant differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

icant only during LASV-ExoN infection, and there was no significant difference between LASV-WT and LASV-ExoN infections. The induction of RIG-I mRNA synthesis in DC was absent for LASV-WT, weak for MOPV, and strong for LASV-ExoN infection. Moreover, the transcription of RIG-I mRNA was higher in LASV-ExoN-infected cells than in LASV-WT-infected cells, and this difference was significant. These results, associated with previous studies that showed that RIG-I signaling is activated by LASV RNA (14), seem to be in favor of an important role for RIG-I in the innate immune response.

We investigated the role of RIG-I in LASV-induced innate immunity by infecting 293T cells with MOPV, LASV-WT, or LASV-ExoN or stimulating these cells with Toll-like receptor (TLR) li-

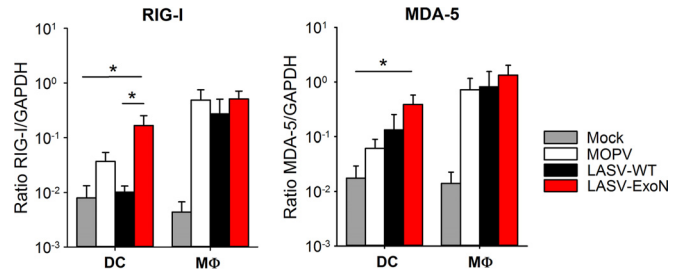


FIG 3 Levels of RIG-I and MDA-5 mRNA in infected DC and MΦ. Total RNA was extracted from cells 24 h postinfection (MOI of 2). cDNA was synthesized with oligo(dT) primers and amplified by quantitative PCR with commercial primers and probes (Applied Biosystems, Life Technologies). Results are expressed as gene/GAPDH ratios and as the means \pm SE of results from 3 independent experiments with cells from different donors. Mann-Whitney tests were carried out for statistical analysis. Significant differences are indicated by asterisks (*, $P < 0.05$).

gands (LM-MS [lipomannan from *Mycobacterium smegmatis*] for TLR2, pIC for TLR3, and R848 for TLR7/8; all from Invitrogen). Cell activation was observed only in response to LASV-ExoN, which induced the synthesis of large amounts of mRNA for IFN- β , TNF- α , and CXCL10. The 293T cells did not respond to any of the TLR ligands tested, suggesting that the responses observed in subsequent experiments were not due to a TLR response (Fig. 4A). Even though TLR2 has been implicated in the response to lymphocytic choriomeningitis virus (LCMV) and MOPV (21) and the involvement of other TLRs recognizing RNA, such as TLR7, has also been suggested (22), we focused here on the RIG-I-mediated response.

We transfected 293T cells with control or RIG-I small interfering RNA (siRNA) and then carried out mock infections or infected the cells with LASV-ExoN 24 h afterwards. We analyzed mRNA synthesis 24 h after infection. LASV-ExoN infection resulted in the synthesis of large amounts of RIG-I mRNA (Fig. 4B). Transfection with the control siRNA had no significant effect, whereas transfection with the RIG-I siRNA resulted in much lower levels of RIG-I mRNA. The levels of mRNA for type I IFN, TNF- α , and CXCL10 in infected 293T cells transfected with RIG-I siRNA were much lower than those in control siRNA-transfected cells (Fig. 4B). This pattern was particularly marked for IFN- α 1 and - α 2, as mRNA levels for these molecules were similar to the basal levels observed in mock-infected nontransfected cells. These results confirm the crucial role of RIG-I in the innate immune response induced by LASV-ExoN.

Other groups have identified NP as the only arenavirus protein able to inhibit the type I IFN response. The role of ExoN activity in the inhibition of IRF-3 translocation and, consequently, in the inhibition of the IFN- β - and NF- κ B-mediated responses has been demonstrated *in vitro* with recombinant NP (10, 11, 23). We provide here the first demonstration of the crucial role of the ExoN function during infection. The abolition of ExoN activity leads to full activation of the innate immune response and to apoptosis after the infection of DC and MΦ, the principal targets of the virus. These results suggest that LASV NP inhibits the recognition of viral RNA by RIG-I, thereby concealing the infection from host sensors. The strong activation and maturation of APC induced by LASV-ExoN infection indicate that the immunosuppressive properties of LASV are essentially due to the ExoN function of NP. This use of ExoN activity for immune evasion is particularly efficient, as acti-

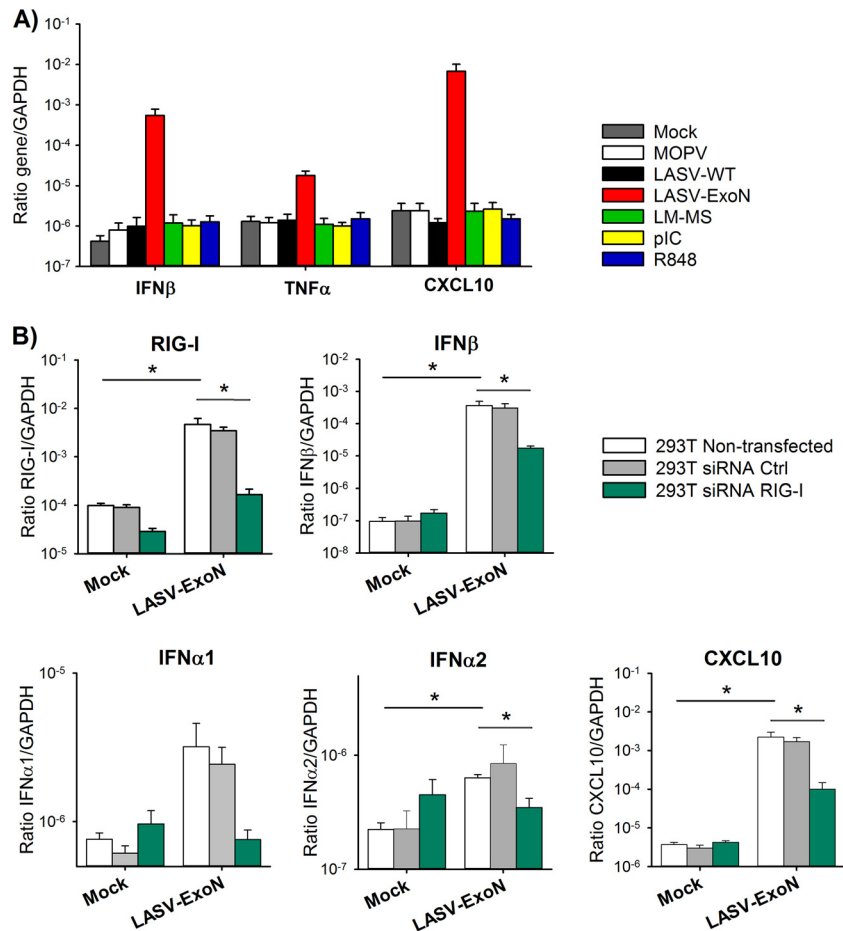


FIG 4 Role of RIG-I in cell activation during LASV-ExoN infection. (A) 293T cells were infected at an MOI of 2 or stimulated with TLR ligands (all from InvivoGen). LM-MS activates TLR2, p(I-C) activates TLR3, and R848 activates TLR7/8. The efficacies of the ligands were tested on 293T cells transfected in a stable manner with constructs encoding these TLRs (data not shown). Cells were lysed 24 h after infection or after stimulation, and total RNA was extracted. cDNA was synthesized with oligo(dT) primers and amplified by quantitative PCR, as previously described. The gene/GAPDH ratios were calculated and are expressed as the means \pm SE of results from 3 independent experiments. (B) We assessed the role of RIG-I in LASV-ExoN infection by transfecting 293T cells 24 h before infection (MOI of 1) with control (Ctrl) siRNA or RIG-I siRNA. Cells were lysed 24 h postinfection, and total RNA was extracted. cDNA was synthesized with oligo(dT) primers and amplified by quantitative PCR, as previously described. The gene/GAPDH ratios were calculated and are expressed as the means \pm SE of results from 4 independent experiments. Mann-Whitney tests were carried out for statistical analysis. Significant differences are indicated by asterisks (*, $P < 0.05$).

vation was completely abolished in APC infected with LASV-WT. Moreover, the stronger immunogenicity of LASV-ExoN than of MOPV suggests that the ExoN domain, which is also present in the MOPV NP, probably at least partly inhibits the induction of type I IFN production. Indeed, LASV and MOPV NP sequences share the same residues in the DEDDh motif, responsible for ExoN activity (data not shown). Finally, it is unlikely that ExoN is involved exclusively in innate immune system evasion, and further investigations of its role in viral replication are required.

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